Wilhelm Roux's Archives 185, 95–98 (1978)

Roux's Archives of Developmental Biology

Short Communication

Chitin Biosynthesis in Imaginal Discs Cultured in vitro*

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Summary. We have investigated the stimulation of cuticle production by imaginal discs of *Plodia interpunctella* in tissue culture. We turned to biochemical methods to assess the quantitative effects of beta-ecdysone on chitin biosynthesis in wing discs incubated with 0.5 μC of C^{14}-glucosamine for the final 24 h of culture.

We demonstrated that imaginal discs of *P. interpunctella* respond to increasing concentrations of β-ecdysone with increased synthesis. The threshold is between 0.01 and 0.1 μg/ml of hormone (2×10^{-8} M to 2×10^{-7} M). These data represent the first demonstration of quantitative biosynthesis of chitin by a developing tissue in vitro in relation to varying amounts of hormone. Additionally, protein synthesis during the β-ecdysone-dependent period was necessary for chitin synthesis. This system thus lends itself to a detailed investigation of the control of chitin biosynthesis.

Key words: Ecdysone – Imaginal Disc(s) – Chitin biosynthesis – Tissue culture – In vitro.

Because of the central role of the integument in the physiology and development of insects there has been an increased focus on the chemical and physical properties of the cuticle (e.g., Neville, 1975; Hepburn, 1976). This interest has been heightened with the advent of pesticides that interfere with chitin synthesis (Post et al., 1974). Surprisingly, there is still relatively little known about the biosynthesis of chitin in insects. In contrast, there has been substantial progress in understanding the production of this abundant structural material in yeasts and fungi where cell-free systems have been used to great advantage in determining the role and control of chitin synthetase (Cabib et al., 1974). Thus far there have been no published reports of cell-free synthesis of insect chitin.

* We wish to dedicate this paper to the memory of a fine colleague and friend, Dr. Andrzej Dutkowski

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0340-0794/78/0185/0095/$01.00

A central problem in the investigation of ecdysone-induced chitin synthesis in tissue culture has been the small amount of material available for assay, and the consequent difficulty of conducting quantitative experiments with a product proven to be chitin. Thus, there is little information on the dynamics of chitin synthesis in tissue culture. Some of the barriers to progress in this area have been removed by the recent work of Marks and Sowa (1976) and of Vardanis (1976). They demonstrated that orthopteran tissue produces chitin in vitro and that this can be monitored by acceptable biochemical methods.

In our laboratory we have investigated the stimulation of cuticle production by imaginal discs of *Plodia interpunctella* in tissue culture. We have reported previously that in response to *beta-ecdysone,* cultured wing discs secrete a complete chitin-containing cuticle (Oberlander and Leach, 1975; Dutkowski, et al., 1977). Further, we showed that the stimulation of cuticle deposition required protein synthesis during the ecdysone-dependent period of culture (Oberlander, 1976). We have turned to biochemical methods to assess the quantitative effects of beta-ecdysone on chitin biosynthesis in cultured wing discs. We have used whole wing discs because our attempts to measure chitin synthetase activity in a cell-free system have not been successful. Secondly, we have inquired whether protein synthesis is a requirement for the synthesis of chitin in cultured wing discs. This work demonstrates the feasibility of using cultured wing discs as a model system for investigating chitin biosynthesis in insects.

Materials and Methods

The Indian meal moth, *Plodia interpunctella* was reared on a cereal diet by the procedures of Silhacek and Miller (1972). Mesothoracic wing discs from final instar larvae (15–18 mg) were dissected, rinsed, and cultured in modified Grace's medium as previously described (Dutkowski and Oberlander, 1974). The paired discs were divided between experimental and control dishes or between 2 experimental dishes as appropriate. Each culture dish contained 20 wing discs. Treatment with β-ecdysone did not begin until the discs had been cultured in vitro for 24 h.

To measure chitin biosynthesis, the wing discs were incubated with 0.5 μC of C^{14}-glucosamine (sp. act. 0.25 mc/mM) for 24 h. The chitin was then isolated from the discs on glass fibre filters by the procedure of Vardanis (1976), and the radioactivity measured in a Packard (2450) Tricarb liquid scintillation spectrometer. The counting efficiency was 79%; quenching was monitored with an external standard. Linear uptake and incorporation of the isotope by cultured *P. interpunctella* discs has already been reported (Oberlander and Leach, 1975). In the present experiments we demonstrated that the isotope was incorporated into chitin by first trapping the radiolabelled material on glass filters followed by incubation with and without chitinase (10 mg/ml 0.05 M acetate, pH 5.2) at 37° C for 48 h. Subsequent filtration and counting of the incubated samples revealed a 95% reduction in radioactivity trapped on the chitinase-treated sample relative to the untreated chitin sample.

Results and Discussion

The first objective of these experiments was to establish a time-course for chitin biosynthesis following treatment with β-ecdysone. Figure 1 shows the results of incubation of wing discs with C^{14}-glucosamine following a 24-h pulse of β-ecdysone (0.5 μg/ml). The '0' day cultures are shown separately though neither "control" nor "experimental" dishes contained ecdysone. Day '1' cultures

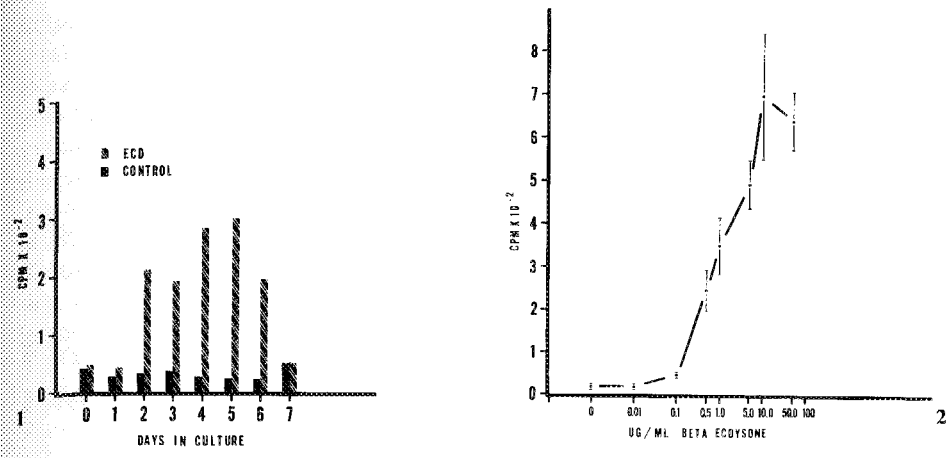

Fig. 1. Incorporation of C^{14}-glucosamine into chitin in wing discs cultured in vitro with (ECD) and without (CONTROL) 0.5 µg beta-ecdysone/ml medium for various incubation periods. The beta-ecdysone incubation consisted of a 24-h pulse on day 1 of culture. The data are the result of a 24-h incubation with isotope beginning on the day of culture indicated in the figure

Fig. 2. Incorporation of C^{14}-glucosamine into chitin in wing discs cultured in vitro with various concentrations of beta-ecdysone. The beta-ecdysone incubation consisted of a 24-h pulse after one day of culture. The 24-h isotope incubation began when the discs were transferred to new medium at the conclusion of the hormone treatment

were exposed to β-ecdysone and C^{14}-glucosamine simultaneously. Older cultures (following the β-ecdysone pulse) received isotope on the day indicated. Clearly, there was a marked stimulation (about 700% vs. control) in chitin synthesis during the second day following addition of hormone. By contrast our earlier study of this system revealed only a 50% increase in counts in the entire imaginal disc (Oberlander and Leach, 1975). Thus, isolating the chitin from the discs permitted a greater sensitivity in demonstrating a hormonal effect. The elevated incorporation levels are maintained for a five-day period before falling to control levels. During this period the lamellate endocuticle is laid down (Dutkowski et al., 1977). The fall in incorporation coincides with the appearance of complete tanned cuticle (Oberlander and Leach, 1975).

We next demonstrated that *P. interpunctella* imaginal discs respond to increasing concentrations of β-ecdysone with increased synthesis (Fig. 2). The threshold is between 0.01 and 0.1 µg/ml of hormone (2×10^{-8} M to 2×10^{-7} M). The isotope incorporation rate levels off between 10.0 and 50.0 µg/ml. From 0.1 to 10.0 µg/ml the level of incorporation is proportional to the log of the β-ecdysone concentration. These data represent the first demonstration of the quantitative biosynthesis of chitin by a developing tissue in vitro in relation to varying amounts of hormone.

Simultaneous exposure of cultured wing discs to β-ecdysone and cycloheximide, a known inhibitor of protein synthesis, blocked the subsequent appearance of cuticle (Oberlander, 1976). In the present experiments we measured C^{14}-glucosamine incorporation into chitin 2 days after incubation of the discs with

β-ecdysone (0.5 μg/ml–24 h) and cycloheximide. A concentration of 10.0 μg/ml of cycloheximide reduced chitin synthesis by 60% and protein synthesis (H^3-leucine incorporation into TCA-precipitable material) by 80%. These results suggest that protein synthesis during the ecdysone-dependent period is necessary for subsequent chitin synthesis. However, there is the possibility that a secondary action of cycloheximide may be involved, since lower doses (1.0 and 5.0 μg/ml) reduced chitin synthesis without comparable reductions in protein synthesis.

We conclude from the time-dose relations that β-ecdysone stimulated chitin production in *P. interpunctella* imaginal discs in vitro. Additionally, protein synthesis during the β-ecdysone-dependent period was necessary for chitin synthesis. This system thus lends itself to a detailed investigation of the control of chitin biosynthesis in insect tissues.

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Received April 26, 1978/Accepted May 23, 1978